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VACUUM-ACID HYDROLYSIS OF FUNGAL PROTEIN AND OF OTHER PROTEIN SOURCES

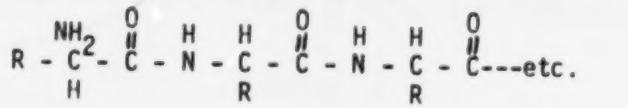
A Division of Research and Development
Open-File Report (RS-03-68-17)

written by
W. Emile Coleman, Research Chemist

U.S. ENVIRONMENTAL PROTECTION AGENCY
Solid Waste Management Office
1971

DISCUSSION

All proteins yield amino acids when hydrolyzed, since a protein molecule may contain hundreds or thousands of amino acid groups. The amino acids are united through an acid amide type of bond called a peptide linkage (-C-N-). The nature of the peptide linkage between the amino acids of the protein is shown below.



Just as is the case with acid amides, the peptide linkages in proteins are resistant to hydrolysis and require prolonged boiling with relatively strong acids or alkalies for complete liberation of each amino acid. The acids most commonly used are hydrochloric (6N), sulfuric (8N), and hydriotic (57%). Among bases, 5N NaOH and 14% $Ba(OH)_2$ are commonly used. Acid hydrolysis of proteins is generally preferred. Alkaline hydrolysis although efficient in liberating the amino acids partly converts the pure optically active forms which exist in proteins into racemic mixtures (mixtures of D and L forms).[1] Acid hydrolysis is essentially free from this objection. Both acid and alkaline hydrolysis lead to some decomposition and loss of the more unstable amino acids.

The developed technology for producing fungal protein in the Bureau of Solid Waste Management's Research and Development Laboratories is the result of research efforts directed toward recycling both starchy and cellulosic wastes.[2, 3] The quality of the protein produced by fermenta-

tion depends upon the amino acid profile which was determined by a quantitative amino acid analysis with an Automatic Amino Acid Analyzer.[4] However, prior to an analysis, hydrolysis of the protein must take place to produce individual amino acids.

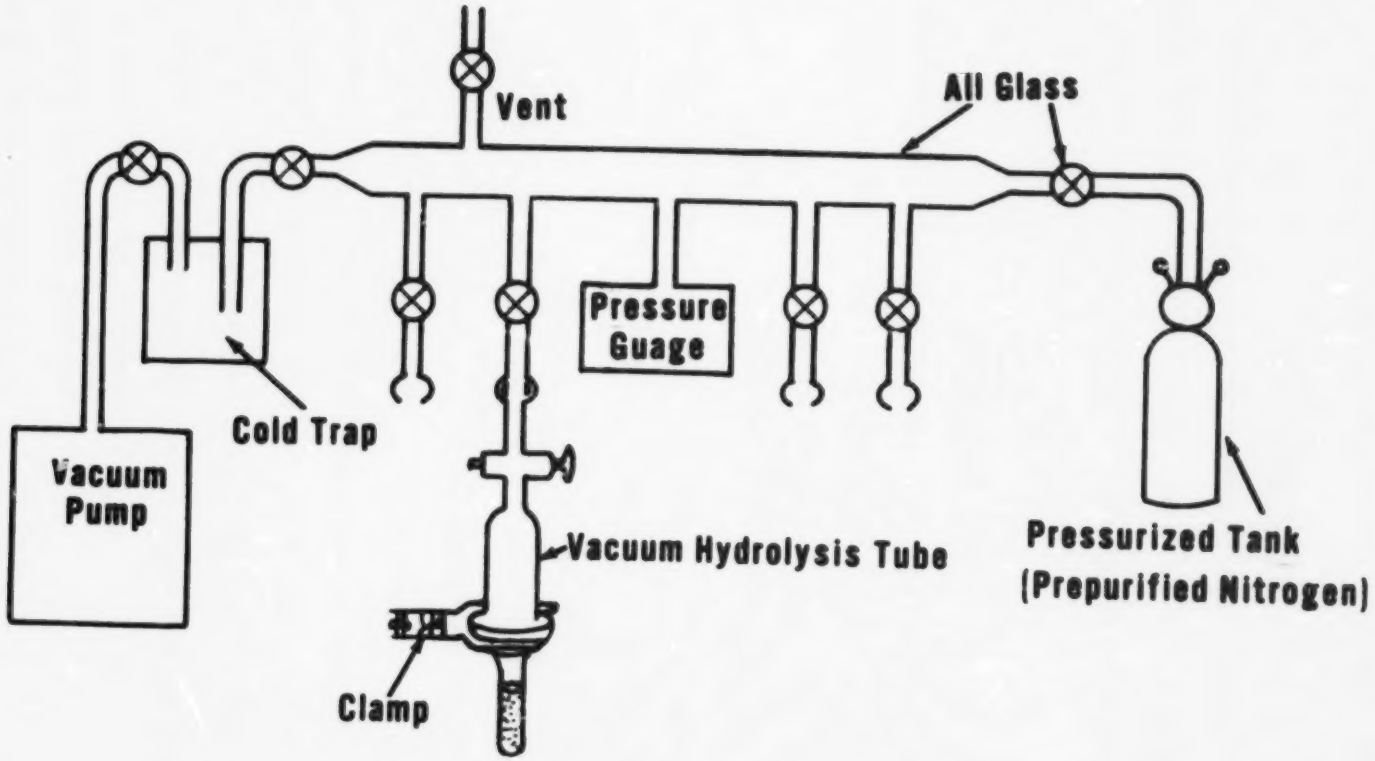
The subject procedure uses acid hydrolysis under vacuum conditions. The use of a vacuum eliminates the oxidizing atmosphere which would result in oxidation or loss of the amino acids. This hydrolysis procedure can be applied to any protein bearing material, plant or animal.

APPARATUS

1. Vacuum pump, capable of obtaining 50-100 microns of pressure.
2. Pressure gauge, range from 50 microns to 1 atmosphere.
3. Cold trap.
4. Dewar flask, containing dry ice/acetone.
5. Evaporating dishes, (1/sample).
6. Glass funnels, (1/sample).
7. Filter paper, #40.
8. Constant temperature oven, thermostatically controlled at 100°C ± 1°C.
9. All glass pressure system as shown in Figure 1.
10. Analytical balance.
11. Pipets, 10 ml., (1).
12. Vacuum hydrolysis tubes (1/sample).[4]
13. Hi-Vacuum grease.

REAGENTS

1. Hydrochloric acid, reagent grade.
2. Prepurified nitrogen, cylinder.



Pressure System for De-Aerating Samples

FIGURE 1

Preparation of Solutions.

Hydrochloric acid - 6N: Add equal volumes of distilled water and concentrated HCl. One liter of solution is sufficient.

SAFETY PRECAUTIONS

The fungal growths when presented to the analyst for protein analysis are usually still active, "alive." Before attempting analysis the molds (fungal growths) should be autoclaved for 15-20 minutes at 120°C.

SAMPLE PREPARATION

The fungal protein is usually grown in a 500 ml. flask containing 100 ml. of growth media. Prior to analysis, the fungal mass is treated as follows:

PROCEDURE	COMMENTS
1. After the fungal mass has been autoclaved, decant the growth media and retain the mold in the flask.	1. The growth media can be discarded.
2. Add approximately 200 ml. of distilled water to the flask.	2, 3, 4. This washing procedure is vital because it assures removal of all salts remaining from the growth media. The metal ions in the growth media, if not removed, become bonded to the cation exchange resin in the chromatographic column and thus interferes with the analysis. Such a condition adversely affects
3. Wash the mold in distilled water by shaking for about five minutes, then decant the liquid.	
4. Repeat steps 2 and 3 twice.	

the capacity of the resin to attract amino acid molecules, thus resulting in loss of resolution. Then, too, the ammonium ion, an ingredient of the media, if not removed by washing produces a huge peak that covers up several of the amino acids on the chromatogram.

5. After the last washings are decanted, pour the mold onto a funnel containing coarse filter paper.

5. Whatman 40 filter paper will suffice.

6. Transfer the mold from the filter paper into an evaporating dish.

6. Shaking the paper will dislodge the mold.

7. Place evaporating dish in an oven thermostatically controlled at 75°C for 24 hours.

7. The analyst should plan his time so that this step can be accomplished by 9:00 am.

8. At the end of 24 hours obtain and record the dry weight of the fungal protein.

9. With mortar and pestle, grind the dried mold until uniform in size.

DE-AERATION AND HYDROLYSIS

PROCEDURE	COMMENTS
1. Weigh out 10-20 mg of the dried protein material into the bottom section of a re-usuable vacuum hydrolysis tube.	1. The two-section hydrolysis tube can be seen in figure 1.
2. Pipet 10 ml. of the 6N HCl into the hydrolysis tube.	
3. Lightly coat the ground glass ball joint and stopcock of the hydrolysis tube with high vacuum grease; then clamp securely.	3. Use Dow—Corning or Apiezon H.
4. Close stopcock on top section of vacuum hydrolysis tube and connect to pressure system as shown in figure 1.	
5. Start the vacuum pump and evacuate pressure system below 50 microns of Hg pressure.	
6. Slowly open the stopcock on the hydrolysis tube to deaerate the mixture. Observe when pressure guage reaches 50-100 microns of Hg pressure.	6. If the stopcock is opened too suddenly, the sample will be pulled into the vacuum lines. A slight tap on the side of the tube will help to dislodge air bubbles when the vacuum is applied.

7. Close the stopcock on the hydrolysis tube and shut off the vacuum from the pressure system.	7. A large stopcock is installed in the system to isolate the vacuum pump.
8. Flush the system with nitrogen for about 3 minutes.	8. Nitrogen pressure at the point of entry to the system is 2-3 PSI.
9. Open the stopcock on the hydrolysis tube and allow tube to fill with nitrogen.	9. Two minutes is sufficient.
10. Close stopcock on vacuum hydrolysis tube and shut off nitrogen supply.	
11. Evacuate system below 50 microns then open stopcock on vacuum hydrolysis tube and evacuate sample down to 50-100 microns.	
12. Repeat steps 7 through 12 twice.	
13. While vacuum pump is running, close stopcock on vacuum hydrolysis tube, then remove tube from vacuum system.	
14. Place vacuum hydrolysis tube in an upright position in a constant temperature oven maintained at 110°C ± 1°C for 22 hours.	

15. At the end of 22 hours remove cample from oven and cool to room temperature.

16. Slowly open stopcock on hydrolysis tube, then remove top section.

17. Filter contents of tube through a medium porosity sintered glass filter under a slight vacuum.

18. Store hydrolyzate in a stoppered bottle.

18. If an analysis of the protein hydrolyzate is not made immediately, store in a freezer.

METHOD EVALUATION

Aliquots of a calibration mixture containing eighteen amino acids, 0.5μ Moles each, were put through the same hydrolysis procedure. Recovery of all the amino acids was quantitative. The percent recovery for all amino acids ranged from 95 to 101.

In order to test the accuracy of the method, three different samples from the same protein source were hydrolyzed using the subject procedure. Three amino acid analyses were run on the protein hydrolyzates and the protein content of each was determined by the summation of the individual amino acids present in each hydrolyzate. The percent protein for the three samples were: 13.30, 13.39 and 13.15.

The interferences most likely to reduce the yield of amino acids in the hydrolyzate have been pointed out in the "comments." Any mineral salts growth media remaining with the mold will greatly reduce the accuracy of an analysis. The presence of undigested starchy and cellulosic substrates in the hydrolysis mixture causes oxidation of the amino acids, thus producing lower yields.

The washing and drying process normally requires a 24-hour period and another 24 hours are required for the de-gasing and hydrolysis process. The analyst could save a day's work if the laboratory submitting the protein sample was instructed as to the washing and drying techniques. The de-gasing and hydrolysis procedures could be started immediately if the sample, upon receipt, was dried and ground.

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